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Phosphoinositide 3-phosphatase segregates from phosphatidylinositol 3-kinase in EGF-stimulated A431 cells and fails to in vitro hydrolyse phosphatidylinositol(3,4,5)*tris*phosphate

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Abstract

Beside 4- and 5-phosphatases playing a role in the interconversion between the D-3 phosphorylated polyphosphoinositides, the only enzyme described so far to be responsible for a phosphomonoesterasic activity on the D-3 position of inositol lipids is a specific 3-phosphatase that hydrolyzes PtdIns(3)P in NIH 3T3 cells. We report here the presence of a potent 3-phosphatase activity in different cell types. This activity is detected both in cytosol and membranes of A431 cells and is inhibited by VO_4^{3-} and Zn^{2+}. Interestingly, the cytosolic activity from A431 cells selectively hydrolyzes in vitro PtdIns(3)P and PtdIns(3,4)P_2, whereas PtdIns(3,4,5)P_3 remains a very poor substrate under the same conditions. Finally, assays of phosphatidylinositol 3-kinase and 3-phosphatase activities in the pool of phosphotyrosine-containing proteins isolated from EGF-stimulated A431 cells suggest a compartmentation of these two antagonistic activities during cell activation.

Key words: Phosphoinositide 3-phosphatase; Phosphatidylinositol 3-kinase; A431 cell; Phosphotyrosine

1. Introduction

The discovery of a link between PtdIns 3-kinase and cellular transformation [1,2] as well as mitogenesis [3] has led to extensive investigations about this lipid kinase and its products, PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. This new signalling pathway is distinct from the classical phosphoinositide turnover since the D-3 phosphorylated inositol lipids are not substrates for PtdIns-specific PLC [4,5]. These observations as well as the time course of their appearance upon cell stimulation led to the suggestion that D-3 phosphorylated inositol lipids may act directly as second messengers. For instance, PtdIns(3,4,5)P_3 has been proposed to play such a role since its production may be correlated with the reorganisation of the cytoskeleton induced by the formyl-Met-Leu-Phe peptide (fMLP) in neutrophils [6]. Recently, it has been suggested that these highly charged D-3 phosphorylated inositol lipids may play a role in down-regulation processes involving vesicle formation and targeting to the endosomal compartment [7–9]. Finally, these phospholipids may also activate downstream signalling enzymes [10], including a PKC isoform [11]. Thus, a number of reports suggest that the products of PtdIns 3-kinase may play important roles in signal transduction. Therefore, the regulation of the synthetic pathway of D-3 phosphorylated inositol lipids has been widely studied these last few years. On the contrary, although potentially of high importance, only very few studies have investigated the degradation pathway of these phosphoinositides [12–14]. A 3-phosphatase that hydrolyses PtdIns(3)P to form PtdIns and inorganic phosphate has been discovered in NIH 3T3 cells [12]. More recently, two different 3-phosphatases have been identified as type I and type II which are able to hydrolyze both PtdIns(3)P and Ins(1,3)P_2 with different affinities [13]. The authors suggested a role of the 3-phosphatase activity in regulating the level of PtdIns(3)P. Such specific dephosphorylation reactions have been reported also in neutrophils [14]; however, in this model the authors investigated in more detail a 5-phosphatase and a 4-phosphatase acting on PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, respectively.

In the present study, we have investigated some characteristics of a phosphoinositide 3-phosphatase activity and its capacity to hydrolyze the different D-3

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Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLC, phospholipase C; PLD, phopholipase D; PtdS, phosphatidylserine; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol(3)phosphate; PtdIns(3,4)P_2, phosphatidylinositol(3,4)*bis*phosphate; PtdIns(3,4,5)P_3, phosphatidylinositol(3,4,5)*tris*phosphate; PtdIns 3-kinase, phosphatidylinositol 3-kinase; SAX, strong anion exchange; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

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phosphorylated inositol lipids. We show for the first time an in vitro selectivity of this enzyme for PtdIns(3)P and PtdIns(3,4)P_2, where as PtdIns(3,4,5)P_3 appears as a very poor substrate for the 3-phosphatase. Moreover, 3-phosphatase was compared to PtdIns 3-kinase for its ability to associate with phosphotyrosine-containing proteins in EGF-stimulated A431 cells. Our data indicate a different compartmentation of the two enzymes during cell stimulation.

2. Materials and methods

2.1. Preparation of cytosol and particulate fraction from A431 cells

A431 cells were grown as previously described [15,16]. They were washed twice with PBS and scraped off at 4°C in a lysis buffer containing 20 mM HEPES (pH 7.35), 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. Cells were lysed using a Downes homogenizer at 4°C, the homogenate was rid of cellular debris by centrifugation at 1,000 × *g* during 10 min at 4°C and the supernatant (lysate) was submitted to a second centrifugation at 100,000 × *g* for 60 min at 4°C. The final supernatant was considered as cytosol while the pellet was resuspended in a small volume of lysis buffer and corresponded to the crude particulate fraction. Proteins were determined using the Bio-Rad Protein Assay Kit (Bio-Rad GmbH, Munich, Germany).

2.2. Immunoisolation of phosphotyrosyl proteins

Immunoisolation was performed using an agarose-linked anti-phosphotyrosine antibody (Oncogene Science) essentially as described [16] except that A431 cells were grown in DMEM with 0.5% (w/v) bovine serum albumin during 12 h before the experiment, then stimulated with 200 ng/ml of EGF for 5 min. Before elution of phosphotyrosyl proteins, the bead matrix was submitted to an additional wash with 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, in order to remove any trace of Nonidet P-40 which would inhibit PtdIns 3-kinase and stimulate PtdIns 4-kinase. Phosphotyrosyl protein elution buffer (15 mM phenylphosphate in 40 mM HEPES (pH 6.8)) did not affect PtdIns 3-kinase and 3-phosphatase activities under our assay conditions.

2.3. In vitro lipid kinase assay and lipid analysis

Immunopurified phosphotyrosyl proteins (100 μl) were incubated for 10 min at 37°C with 50 μM ATP, 2 mM dithiothreitol, 10 mM $MgCl_2$, 10 μCi [γ-^{32}P]ATP (Amersham International) and exogenous lipid vesicles containing PtdIns (50 μM), PtdIns(4)P (50 μM), PtdIns(4,5)P_2 (50 μM) and PtdS (100 μM) as described [19]. The reaction was stopped and lipids were extracted, deacylated and quantified using a HPLC technique on a Whatman Partisphere SAX column, as previously described [17].

2.4. Immunoblotting

Immunodetection of the p85 regulatory subunit of the PtdIns 3-kinase was performed as previously described [18].

2.5. Preparation of radiolabelled D-3 phosphorylated inositol lipids

D-3 phosphorylated inositol lipids were enzymatically synthesized. PtdIns 3-kinase activity was obtained by immunoprecipitation from human blood platelets using an anti-p85 antibody (UBI). Human blood platelets (3.5×10^9 cells/ml) were prepared as previously described [18] and lysed with 1% (v/v) Nonidet P-40 in 20 mM Tris-HCl (pH 7.35), 100 mM NaCl, 5 μg/ml leupeptin and 3 μg/ml aprotinin. Then 5 μl of antibody against p85 were incubated for 1 h at 4°C with 1 ml of the Nonidet-P-40-soluble fraction of human blood platelets. Protein A-Sepharose CL-4B (Sigma) was then added (50 μl) and incubated for 1 h at 4°C. After 4 batch-washing steps with 1 ml of 20 mM Tris-HCl buffer containing 3 μg/ml leupeptin, 3 μg/ml aprotinin and 100 mM NaCl (pH 7.35), immunoprecipitated proteins were incubated for 10 min at 4°C with PtdIns/PtdS vesicles (200 μM PtdIns), 0.5 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl (pH 7.35), 50 μM ATP, 10 μCi [γ-^{32}P]ATP. The reaction was then started by the addition of 10 mM $MgCl_2$ and incubated for 15 min at 37°C. In order to prepare [^{32}P]PtdIns(3,4)P_2 and [^{32}P]PtdIns(3,4,5)P_3, PtdIns(4)P and PtdIns(4,5)P_2 were respectively used instead of PtdIns. The lipid vesicles were in each case unilamellar as previously described [19]. Lipids were immediately extracted by an acidic Bligh and Dyer extraction procedure [20], dried under a nitrogen stream and stored in chloroform/methanol (v/v). Under these conditions more than 95% of ^{32}P-radiolabelled products obtained were D-3 phosphorylated inositol lipids. The cold unphosphorylated phosphoinositides used as substrate for the PtdIns 3-kinase were used as carrier during the 3-phosphatase assay.

2.6. 3-Phosphatase assay

This enzymatic activity was assayed essentially as in [12]. Briefly, radiolabelled substrates (8,000 to 15,000 dpm representing 0.12 to 0.23 picomol per assay) were dried under a nitrogen stream and solubilized in 50 μl of 200 mM KCl, 40 mM HEPES (pH 6.8), 20 mM EDTA, 2 mM EGTA, 1.2% (w/v) octyl glucoside. Reactions were started by addition of the same volume of enzyme, incubated at 37°C during indicated time (10 to 15 min) and stopped by the addition of chloroform/methanol (v/v). Lipids were immediately extracted, deacylated and quantified by HPLC [17], whereas, water-soluble extracts were treated as previously described [21] and analysed by a HPLC technique [17].

3. Results

3.1. Evidence for a potent 3-phosphatase in A431 cells

Picomolar quantities of [^{32}P]PtdIns(3)P with a high specific radioactivity were enzymatically synthesized. This inositol lipid labelled on the D-3 position of the inositol ring was then used as a substrate in order to detect a hydrolyzing activity in A431 cells. Fig. 1a and b show that addition of 100 μg proteins of an A431 cell lysate induced a rapid and total degradation of PtdIns(3)P. The PtdIns(3)P hydrolysis was parallel to an increase of ^{32}P-labelled water-soluble products suggesting that the enzyme responsible for PtdIns(3)P hydrolysis may be: (i) a PLC producing Ins(1,3)P_2; (ii) a PLD producing Ins(3)P; or (iii) a PtdIns(3)P phosphatase releasing $^{32}P_i$. As shown in Fig. 1c, the only ^{32}P-labelled water-soluble compound detected was eluted at the same position as a standard of [^{32}P]-*o*-phosphate, indicating that the enzyme responsible for PtdIns(3)P hydrolysis was a 3-phosphatase. A possible dephosphorylation of Ins(1,3)P_2 or Ins(3)P potentially obtained by PLC or PLD action, respectively, was unlikely since this inositol phosphate 3-phosphatase activity would not function under our assay conditions (100 mM KCl, 0.6% octyl glucoside and carrier PtdIns) [13].

3.2. Properties of the 3-phosphatase from A431 cells

We first compared the 3-phosphatase activity of A431 cells to the one displayed by various cell types. Because of the limited quantity of substrate used in the assay, kinetic constants were calculated according to a first-order kinetic equation as previously described [12]. As indicated in Fig. 2, no dramatic difference could be measured between the different cell types tested. Then, we measured the PtdIns(3)P 3-phosphatase activity in the membranes as well as in the cytosol of A431 cells by

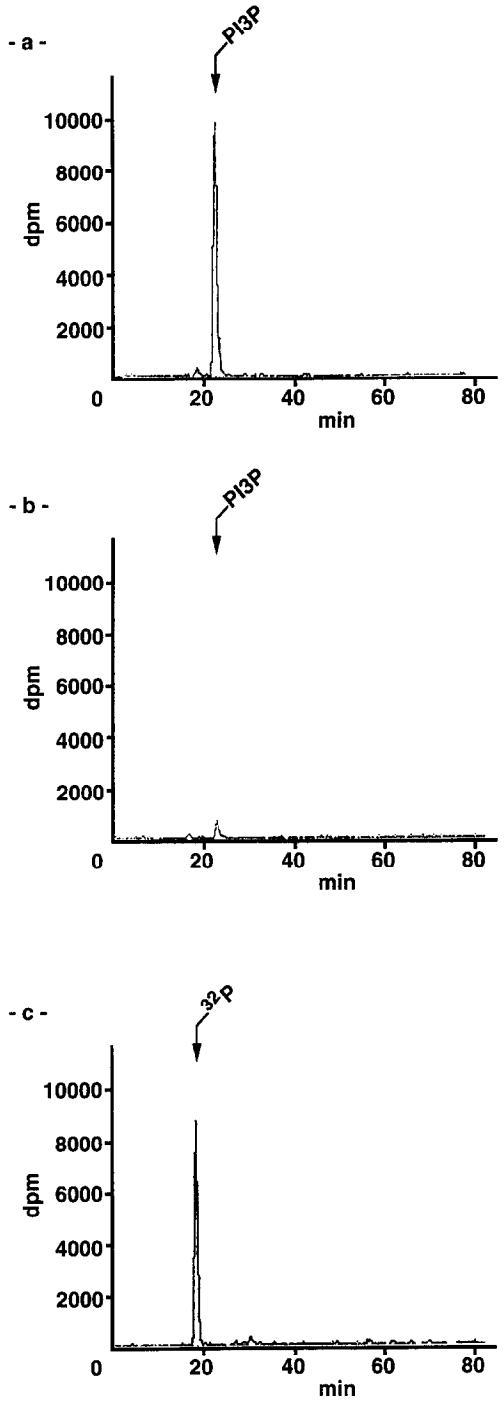

Fig. 1. Hydrolysis of [^{32}P]PtdIns(3)P by A431 cell lysate and HPLC analysis of ^{32}P-labelled water-soluble compounds released. [^{32}P]PtdIns(3)P (10,000 dpm) was incubated during 10 min at 37°C in the absence (a) or in the presence (b) of A431 cell lysate (100 μg proteins) as indicated in section 2. The water-soluble products released during [^{32}P]PtdIns(3)P hydrolysis by the A431 cell lysate were immediately dried under a nitrogen stream, dissolved in 1 ml of water, neutralized and applied to the HPLC anion-exchange column (c). These HPLC profiles were representative of five different experiments with very similar results.

following the production of inorganic phosphate and PtdIns from PtdIns(3)P. On average, 60% of the total cellular activity was recovered in the particulate fraction while 40% was found in the cytosol. This enzyme activity from both cellular compartments was respectively inhibited by 90 ± 12% and 97 ± 8% in the presence of 0.5 mM orthovanadate and of 0.5 mM $ZnCl_2$. Moreover, this enzyme exhibited similar caracteristics toward ions (inhibition by Ca^{2+} and Mg^{2+}, data not shown) as the PtdIns(3)P 3-phosphatase previously described in NIH 3T3 cells [12]. Storage at −20°C for one week slightly reduced its activity (10%). A preliminary experiment indicated that PtdIns(3)P 3-phosphatase activity of A431 cytosol could bind to a column of DEAE-Sepharose and was eluted using an NaCl gradient with a peak of activity released at 0.5 M NaCl. Interesting, as indicated in Fig. 3, beside a dose-dependent PtdIns(3)P hydrolysis by the 3-phosphatase, no PtdIns(4)P degradation could be measured under our conditions by the cytosol of A431 cells. Therefore, we have used this subcellular fraction in order to study the substrate specificity of the 3-phosphatase from A431 cells.

3.3. *In vitro selectivity of 3-phosphatase*

By using different ^{32}P-labelled substrates, we have investigated the specificity of PtdIns(3)P phosphatase present in the cytosol of A431 cells. [^{32}P]PtdIns(3)P, [^{32}P]PtdIns(3,4)P_2 and [^{32}P]PtdIns(3,4,5)P_3 were enzymatically synthesized and used independently or mixed together as substrates for the PtdIns(3)P 3-phosphatase. When the phospholipids were used independently in similar quantities (0.2 pmol per assay), 100 ± 3% (mean ± S.E.M., n = 4) of PtdIns(3)P and PtdIns(3,4)P_2

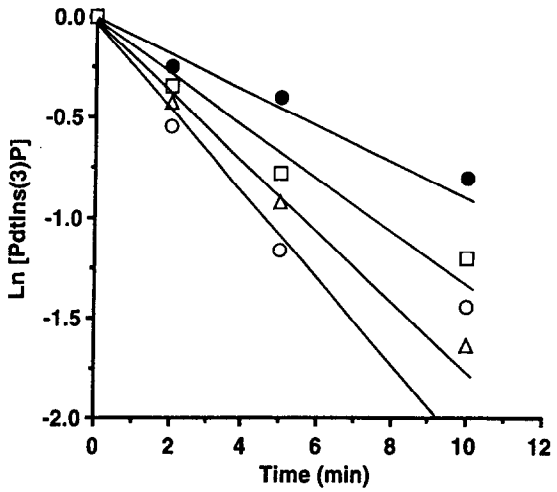

Fig. 2. First-order kinetic analysis of the hydrolysis of PtdIns(3)P by various cell type lysates. Cell lysates (50 μg proteins) were checked for phosphoinositide 3-phosphatase activity according to Lips and Majerus [12]. PtdIns(3)P (14,000 dpm, 2.2 nM) was used for each reaction and assays were performed as described in section 2. (●) A431, (□) HEL-5J20, (△) NIH3T3, (○) human blood platelets. Means ± S.E.M. of the first-order rate constant obtained from two different experiments were: A431, 1.64 ± 0.23 $min^{-1} \cdot mg^{-1}$; NIH3T3, 3.17 ± 0.57 $min^{-1} \cdot mg^{-1}$; HEL-5J20, 2.59 ± 0.39 $min^{-1} \cdot mg^{-1}$; human blood platelets, 4.23 ± 0.83 $min^{-1} \cdot mg^{-1}$.

were hydrolysed by the 3-phosphatase activity present in the cytosol of A431 cells. However, surprisingly, PtdIns(3,4,5)P_3 was almost completely resistant to this enzymatic activity under our conditions (15 ± 8% of hydrolysis, $n = 4$). One may note that [^{32}P]PtdIns(3,4,5)P_3 as well as the other substrates used were homogenously solubilised in the assay buffer containing octyl glucoside. When the ^{32}P-labelled D-3 phosphorylated inositol lipids were mixed together as substrates (Fig. 4a and b), the enzyme could selectively hydrolyze PtdIns(3)P and PtdIns(3,4)P_2 whereas PtdIns(3,4,5)P_3 was again a very bad substrate excluding any possible effect of the cold carrier lipids on the selectivity of the 3-phosphatase. According to the time course experiment (Fig. 4c), in these conditions, 67% of PtdIns(3)P and 70% of PtdIns(3,4)P_2 were hydrolyzed within 2 min whereas at most 15% of degradation of PtdIns(3,4,5)P_3 could be measured even after 15 min.

3.4. *Evidence for a possible differential compartmentation of activated PtdIns 3-kinase and 3-phosphatase during EGF stimulation*

The presence of the PtdIns 3-kinase in the so-called signal transfer particles formed during activation of the cell by different tyrosine kinase activating growth factors has been described extensively [7,22]. In order to determine whether the 3-phosphatase would also be a member of these multienzymatic complexes, they were isolated by the classical antiphosphotyrosine immunopurification. We then compared the PtdIns 3-kinase and 3-phosphatase activities in the pool of immunopurified phosphotyrosyl proteins obtained from resting or EGF-stimulated A431 cells (Table 1). We had previously shown that EGF induced the appearance of PtdIns 4-kinase and PtdIns(4)P 5-kinase in such a fraction [16,23]. By using appropriate assay conditions we show here that EGF treatment induced a significant appearance of PtdIns

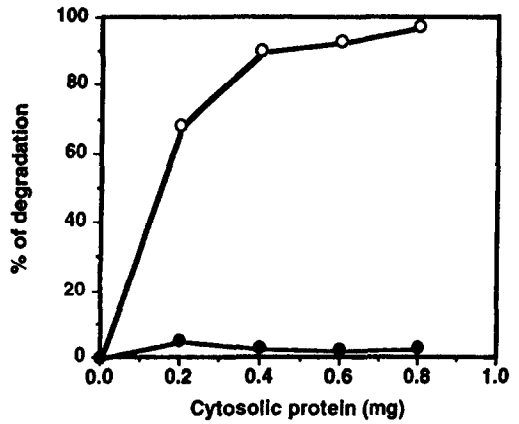

Fig. 3. Degradation of PtdIns(3)P and PtdIns(4)P by increasing amounts of cytosolic proteins. [^{32}P]PtdIns(3)P (10,000 dpm) (○) and [^{32}P]PtdIns(4)P (2,000 dpm) (●) were submitted to hydrolysis by increasing concentrations of A431 cytosolic proteins during 15 min at 37°C. The reaction was stopped and lipids were immediately extracted and quantified by HPLC.

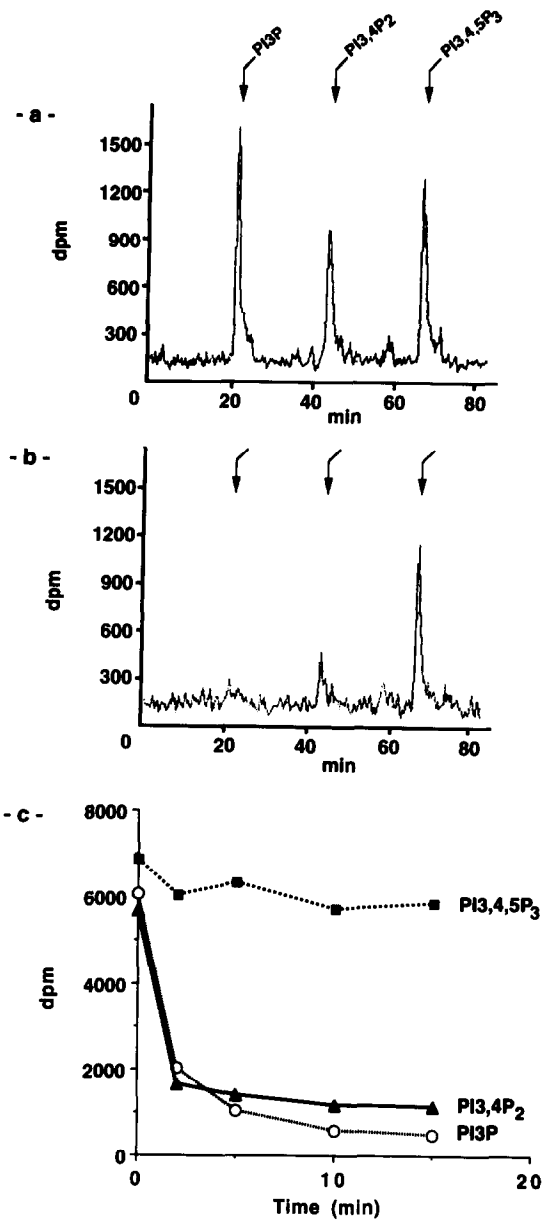

Fig. 4. Substrate selectivity of the 3-phosphatase present in the cytosol of A431 cells. [^{32}P]PtdIns(3)P, [^{32}P]PtdIns(3,4)P_2 and [^{32}P]PtdIns(3,4,5)P_3 were mixed together and incubated in the absence (a) or in the presence (b) of 200 μg of A431 cytosolic proteins during 15 min at 37°C, the products of the reaction were analysed by HPLC. Fig. c represents a time course of degradation of the different D-3 phosphorylated inositol lipids mixed together in conditions similar to those used in Fig. a and b. These results are representative of 3 different experiments.

3-kinase activity in this immunopurified proteins as already described [22], whereas no 3-phosphatase activity was detected in this fraction.

4. Discussion

There is now compelling evidence indicating that PtdIns 3-kinase is an important enzyme in signalling

Table 1
Estimation of PtdIns 3-kinase and PtdIns 3-phosphatase activities in the pool of phosphotyrosyl proteins obtained from resting and EGF-stimulated A431 cells

	Immuno-quantification of p85 (arbitrary unit)[1]	PtdIns 3-kinase activity (fmol of [^{32}P]PtdIns(3)P/min)	PtdIns 3-phosphatase activity (% of [^{32}P]PtdIns(3)P hydrolyzed)
Resting cells	29 ± 10	0.09 ± 0.01	ND[2]
EGF-activated cells	333 ± 27	8.00 ± 0.20	ND

[1] Pixel points determined by densitometric analysor (CRIS, Ramonville, France).
[2] ND: not detected.
A431 cells were grown in 140-mm culture dishes to pre-confuency and were stimulated or not with EGF, phosphotyrosyl-proteins were subsequently immunopurified. PtdIns 3-kinase and PtdIns 3-phosphatase activities were estimated in the pool of phosphotyrosyl-proteins obtained from resting and activated cells. Results for enzyme activities are means ± S.E.M. of 3 independent experiments.

processes [7] and suggesting that its products may play important roles. Thus, their concentration and location within the cell may be strictly controlled by subtle processes regulating synthesis and/or degradation. However, only a very few reports describe a degradation pathway for these D-3 phosphorylated inositol lipids [12–14]. In this study, we have measured in A431 cells and other cell types a phosphatase activity able to degrade PtdIns(3)P by hydrolyzing the monoester phosphate in the D-3 position of the inositol ring.

We show that the 3-phosphatase activity present in cytosol and membranes of A431 cells displays quite similar characteristics to those previously described by Lips and Majerus in NIH 3T3 [12]. Preliminary data indicated that the 3-phosphatase activity measured in this study can bind to a DEAE-Sepharose column as also demonstrated by these authors. Furthermore, when similar quantities of the different D-3 phosphorylated inositol lipids were used independently or mixed together, the 3-phosphatase activity present in A431 cytosol was able to rapidly hydrolyze PtdIns(3)P and PtdIns(3,4)P_2 but very weakly PtdIns(3,4,5)P_3.

Thus, in agreement with Stephens et al. [14], we found that the 3-phosphatase seems to be responsible for a very small proportion of the turnover of PtdIns(3,4,5)P_3. However, our data suggest that, in A431 cells, the 3-phosphatase activity is likely responsible for the main degradation route of PtdIns(3,4)P_2 and PtdIns(3)P. So far, it is difficult to assert that a unique protein is responsible for the degradation of PtdIns(3)P and PtdIns(3,4)P_2 since different enzymes with very similar characteristics may be responsible for their hydrolysis. Although assays using exogenous phospholipids must be cautiously interpreted, our observations suggest that PtdIns(3,4,5)P_3 degradation could be controlled by an enzymatic mechanism different from that responsible for the degradation of PtdIns(3)P and PtdIns(3,4)P_2 and emphasize the fact that this inositol lipid may play an important role in signalling pathways. A 5-phosphatase responsible for the degradation of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 and a 4-phosphatase hydrolyzing PtdIns(3,4)P_2 to PtdIns(3)P have been found in neutrophils using in vitro assays in the absence of detergent [14]. Although these activities may also be present in A431 cells, they were not detected under our assay conditions using detergent.

Finally, we show that, in the opposite of PtdIns 3-kinase, the 3-phosphatase does not belong to the so-called signal particles whose formation is transiently induced by EGF stimulation. Moreover, we could never measure any increase of the 3-phosphatase activity by EGF in A431 cells (not shown). From these data, one can speculate that this enzyme has a high basal activity and that the crucial event for the synthesis of D 3-phosphorylated inositol lipids would then be the transient activation of PtdIns 3-kinase. Further studies will be necessary to elucidate the regulation of the balance between 3-kinase and 3-phosphatase in the control of the level of D-3 phosphorylated inositol lipids.

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